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The Effects of Ethynodiol Diacetate on Epinephrine-Treated Glial-Derived Cells

Jesse D. Hunt

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COLUMBUS STATE UNIVERSITY

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JESSE D. HUNT

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Abstract

Neuroprotective agents, including specific hormones, may play a role in preventing cellular damage following an ischemic event or brain injury. Astrocytes are specialized glial cells within the central nervous system that play roles in synapse development, blood flow regulation, extracellular homeostasis, synapse function, energy and metabolism (Sofroniew and Vinters, 2009). Research using astrocytes in cell viability studies can be beneficial in deepening our understanding of specific neuroprotective effects of various steroid hormones including progesterone. Progesterone has been shown to have neuroprotective capabilities in the central nervous system (Scarpin et al., 2009). Although progesterone shows great therapeutic potential, effects of the synthetic forms, progestins, including ethynodiol diacetate, are not as well known (Asi et al., 2016). The purpose of my study was to understand the effects of progestins on glial cell-derived culture viability and understand how progestins influence the viability of astrocytes under the treatment of epinephrine. My goal was to determine the degree to which *in vitro*-pretreatment of cultured glia with varying concentrations of ethynodiol diacetate affects cell viability and protects cells from the toxicity of epinephrine. Cells treated with epinephrine had significantly higher cell viability than untreated cells and treated cells were not significantly different from untreated controls (2-way ANOVA, $F_{9,30}$=4.478, P=0.043). There was no significant difference in cell viability between concentrations of ethynodiol diacetate (2-way ANOVA, $F_{9,30}$=0.0206, P=0.933). The results of this study suggest that under these treatment parameters, ethynodiol diacetate does not protect glial-derived cultures from oxidative stress. These results also show that short-term epinephrine treatment improves cell viability in the human glioma-derived cell line used in this study.

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Dedications

Dear Justin: You have played the biggest role in my success in college and in life. I am truly grateful for your continuous support in everything I do.

Dear mom, dad, and Gary: I thank you for raising me to be a strong and independent woman and for always pushing me to be my best. I love you guys so much and would not be the woman that I am today without you.

Dear Tracee: Thanks for always being around when I need you most. You are my right-hand man and I would not have made it through these degrees without you.

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Background

Oral contraceptive pills are composed of estrogen and progestogens or progestogens alone. Progestogens prevent pregnancy using three paths of prevention: (1) inhibition of ovulation, (2) thickening of cervical mucus to prevent sperm penetration, and (3) changes in endometrial lining to prevent implantation (Regidor, 2018). Current forms of contraceptives include combined hormonal contraceptives in the form of pills, patches or vaginal rings, and progestogen-only contraceptives in the form of pills, injections, implants, or intrauterine devices (Regidor, 2018). The most frequent side effect of progestogen-only contraceptives is irregular bleeding, but other side effects include menstrual irregularities, abdominal discomfort, dizziness, anxiety, asthenia, breast tenderness, mood changes, mild hirsutism, sexual pain, acne, headache, weight gain, mastalgia, vaginal infections, and deleterious interactions with antibiotics (Regidor, 2018). Progestogen-only contraceptives are recommended for women that are breastfeeding, smoking, obese, hypertensive, and for women who have a history of heart disease, migraines, and cancer (Ahrendt et al., 2010).

Under normal circumstances, progesterone is a hormone that prepares the female body for pregnancy. The term progestogen is used to describe a substance that mimics progesterone by binding to progesterone receptors (PR) (Carroll et al., 2016). Progestogens include natural progesterone produced by the ovaries and synthetic progestins that are taken as contraceptives or hormone replacement therapy (HRT). Synthetic progestins have a different chemical structure from natural progesterone, but replicate some of the same physiological effects (Asi et al., 2016). When fertilization occurs, progesterone thickens the uterus lining for implantation and nourishment of the ovum (Lieberman and Curtis, 2017). Progesterone also causes the mammary glands to dilate and develop into secretory epithelium (Lieberman and Curtis, 2017). Along with preparing the body for pregnancy, progesterone prevents further ovulation after an ovum has been

formed (Sitruk-Ware and El-Etr, 2013). Since progesterone is derived from cholesterol, it is a hydrophobic substance and does not readily enter the bloodstream when taken orally. Progestins are synthetic versions of progesterone that have hydrophilic functional groups added to better be absorbed by the body and into the bloodstream (Berg, 2015). Progestins can be divided into two classes based on the chemical similarity to either testosterone or progesterone. Progestins, unlike progesterone, bind to androgen receptors, estrogen receptors, glucocorticoid receptors, and mineralocorticoid receptors with different affinities. These interactions may be agonistic, antagonistic, or have no effect (Giatti et al., 2016).

Progesterone and progestins affect the cell by binding to nuclear progesterone receptors (PR). PR are ligand-activated nuclear transcription regulators which are made up of a central DNA binding domain and a carboxyl-terminal ligand binding domain (Scarpin et al., 2009). These receptors also contain activation and inhibitory functional elements. PR regulates transcription through genomic and non-genomic pathways. There are two PR isoforms: PRA and PRB. PRA differs from PRB in that it lacks 164 amino acids at the N-terminus. These isoforms have been shown to differentially regulate gene transcription, although the mechanism has not been fully studied. In the mouse, PRB was shown to be necessary for the development of mammary glands, and PRA was necessary for the development of uterine and reproductive tissues (Scarpin et al,. 2009). PR proteins are expressed in tissues including the uterus, mammary gland, brain, pancreas, bone, ovary, testes, and urinary tract (Scarpin et al,. 2009).

Along with progesterone's role in reproduction, it also has other therapeutic potentials in the body including the cardiovascular system, bone tissue, and central nervous system (Scarpin et al., 2009). PRs have been found within many areas of the brain and have been associated with functions including cognition, neuroprotection, and dendritic remodeling. Specifically, progesterone has also been shown to reduce epileptic activity and has neuroprotective abilities

after traumatic or ischemic brain injuries (Sitruk-Ware and El-Etr, 2013). Although progesterone shows great therapeutic potential, the effect of progestins on the nervous system varies greatly based on the structure of the progestin. Some progestins, such as segesterone acetate, have been observed to promote remyelination of neurons by oligodendrocytes, while others such as medroxyprogesterone acetate did not exhibit the same protective effects (Giatti et al., 2016). Due to the lack of knowledge of the effects of progestins on the central nervous system, it is necessary that the effects of progestins on the central nervous system be studied.

Astrocytes are specialized glial cells within the central nervous system (CNS) that outnumber neurons with a ratio of 5:1. They perform a variety of functions that are critical for the brain and spinal cord and are found within the entirety of the CNS. Astrocytes play roles in synapse development, blood flow regulation, extracellular homeostasis, synapse function, energy and metabolism, and are main components of the blood-brain barrier (Sofroniew and Vinters, 2009). Due to their participation in extracellular metabolism, astrocytes can remove harmful toxins from the CNS. Astrocyte deficiency or damage has been linked to neurodegenerative diseases such as Alzheimer's disease and dementia because of the inability to remove these toxins (Chen et al., 2003). Although astrocytes show neuroprotective capabilities, they have also been shown to trigger inflammatory responses and increase neuronal apoptosis (Zhang and Jiang, 2015). Research using astrocytes in cell viability studies can deepen our understanding of specific neuroprotective effects of progestins.

Although progesterone is known to have several neuroprotective properties in the nervous system, the effects of progestins vary within the system and have not been extensively studied (Sitruk-Ware and El-Etr, 2013). The purpose of my study was to understand the effects of progestins on the viability of glial-derived cells and understand how progestins influence the viability under the stress of epinephrine. The progestin used in this study is ethynodiol diacetate,

which is chemically-related to testosterone (Giatti et al., 2016). Along with having some androgenic effects, this progestin also exhibits slight estrogenic effects, but its role in the CNS is unknown. My goal was to determine the degree to which *in vitro*-pretreatment of cells with varying concentrations of ethynodiol diacetate affects cell viability and protects cells from the toxicity of epinephrine. Cells were treated with ethynodiol diacetate, and half of the treatment groups were stressed using epinephrine. I predicted that the cell viability would decrease following epinephrine treatment alone as a result of the stress treatment. I predicted that ethynodiol diacetate pretreatment of the cells prior to epinephrine treatment would result in increased cell viability compared to untreated epinephrine-stressed cells.

Methods

Cells

Cells used in this research were human cultured glioma-derived glial cells (1321N1) purchased from Sigma Aldrich. These cells demonstrate astrocyte-like functions and properties in culture. The cell media used was Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% streptomycin/penicillin in T-75 flasks. The media was changed every 2-3 days. After the cells propagated to about 70-80% confluency, subculturing started. A solution of 0.53 mM EDTA and 25% trypsin was added to the flask (2-3 mL). Complete growth medium was added after the cells had dispersed and was followed by cell aspiration. The cells were centrifuged to remove excess trypsin and plated in a 96 well plate. Each well contained 200 µL of media with a concentration of $1x10^6$ cells/mL. The cell concentration was determined using a BioRad cell counter. Using equal parts trypan blue and prepared astrocytes, a solution was made. Next, 10 µL of this 1:1 solution was added to a BioRad cell counter slide and inserted into the cell counter. Live cell counts were obtained and used for concentration adjustment to 1 x 10^6 cells/mL and then added to wells. Treatments started the following day. Cells were stored at 37°C with 5% supplemented CO_2.

Treatment of Cells

Cells in culture were treated with varying concentrations of ethynodiol diacetate (0 M control, 10 nM, 100 nM, 1 µM, 10 µM) (Johnson, 2016). Half of the wells were treated with epinephrine (200 µM) 60 minutes after ethynodiol diacetate treatment (Smith, 2017). This concentration of epinephrine was chosen based on a previous study that stated 100 µM epinephrine treatment had no effect on the cell viability of astrocytes. The author stated that, consistent with previously published studies, increased concentration of epinephrine should stress the cells (Smith, 2017). The media was changed one hour after epinephrine addition, and ethynodiol diacetate was added

to the new media. Viability of the astrocytes was measured after 24 hours. Four trials were completed of these above treatments. The treatments are outlined in Table 1.

Treatment	**Progestin Concentration**				
Ethynodiol Diacetate	Control (no progestin)	10 nM	100 nM	1 μM	10 μM
Ethynodiol Diacetate + Epinephrine	Control (no progestin)	10 nM	100 nM	1 μM	10 μM

***Epinephrine:** 200 μM

Table 1. 96-well plate diagram for treatment. Cells in the first row were treated with ethynodiol diacetate in concentrations of 0 M, 10 nM, 100 nM, 1 μM and 10 μM. Cells in the second row were treated with the same concentrations of ethynodiol diacetate and were also treated with 200 μM epinephrine one hour after initial ethynodiol diacetate treatment. Two hours after initial ethynodiol diacetate treatment, media was changed and cells were retreated with ethynodiol diacetate.

MTT Cell Proliferation Assay

Cell viability after treatment was assessed using an MTT Cell Proliferation Assay (Sigma Aldrich) after 24 hours of treatment. A blank well was included that contained only media with no cells. To each well, 10 μL of the MTT reagent was added and the cells were incubated for three hours and 45 minutes or until a blue/purple precipitate formed. Next, 100 μL of the MTT reagent was added to the wells. The spectrophotometric plate reader was used to measure absorbance at 570 nm. A high absorbance value indicated high cell proliferation.

Analysis of data

Four trials were run. To analyze absorbance values, the blank reading was subtracted from the treatment readings and a ratio made with the corrected control value. The independent variables that were measured were ethynodiol diacetate treatments and if astrocytes were stressed or not

stressed. To evaluate statistical significance between treatments, a 2-Way Analysis of Variance (ANOVA) and Tukey's post hoc test was used with a p-value<0.05 indicating significance.

Results

There was significant increase in the viability of cells treated with epinephrine compared to untreated cells (Figure 1, 2-way ANOVA, $F_{9,30}$=4.478, P=0.043). There was no significant difference in astrocyte cell viability between concentrations of ethynodiol diacetate or between cells treated with ethynodiol diacetate and untreated cells (Figure 1, 2-way ANOVA, $F_{9,30}$=0.0206, P=0.933). Cell viability data are reported as the average absorbance in the treatment well compared to control (+/- standard deviation). The viability ratio of the cells treated with 0 M ethynodiol diacetate followed by epinephrine treatment was 11.49 (+/- 10.71). The viability ratios of the cells treated with 0.01 μM ethynodiol diacetate were 3.82 (+/- 3.94) and 4.67 (+/- 4.54) for the cells untreated with epinephrine and cells that were treated with epinephrine, respectively. The viability ratios of the cells treated with 0.1 μM ethynodiol diacetate were 3.37 (+/- 1.88) and 5.94 (+/- 8.11) for the cells untreated with epinephrine and cells that were treated with epinephrine, respectively. The viability ratios of the cells treated with 1 μM ethynodiol diacetate were 3.01 (+/- 1.88) and 6.28 (+/- 4.76) for the cells untreated with epinephrine and cells that were treated with epinephrine, respectively. The viability ratios of the cells treated with 10 μM ethynodiol diacetate were 3.75 (+/- 3.65) and 4.44 (+/- 5.00) for the cells untreated with epinephrine and cells that were treated with epinephrine, respectively. Figure 1 shows the average viability ratios compared to control (+/- 1 S.D.) of stressed with epinephrine or unstressed, treated with varying concentrations of ethynodiol diacetate.

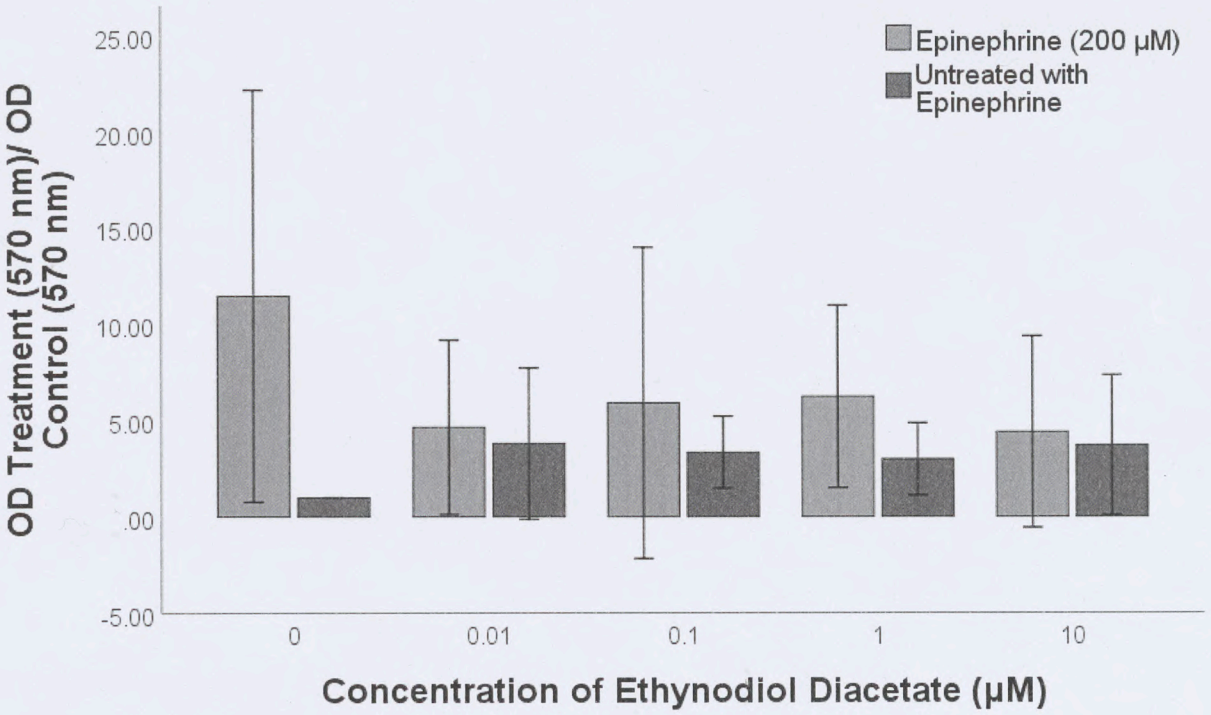

Figure 1. Average astrocyte viability (+/- 1 S.D.) for cells stressed or unstressed when treated with varying concentrations of ethynodiol diacetate. There was no significant difference in astrocyte viability between varying concentrations of ethynodiol diacetate (2-way ANOVA, $F_{9,30}$=0.0206, P=0.933). There was significant increase in glial cell viability (+/- 1 S.D.) between cells that were treated with epinephrine versus the untreated cells (2-way ANOVA, $F_{9,30}$=4.478, P=0.043).

Discussion

There was no significant difference in glial cell viability between ethynodiol diacetate treatment groups. There was a significant difference in cell viability between cells that were stressed with epinephrine and cells that were left unstressed. Cells stressed with epinephrine had increased cell viability than cells that were left unstressed, indicating that epinephrine did not effectively stress the astrocytes and in fact gave the cells a survivability advantage. In previous studies, it was shown that cells derived from cancer cell lines had increased cell viability when treated with epinephrine (Pu J et al., 2012). The cell line used in this study is derived from glioma tissue, which is a cancerous tissue. Thus, this result is consistent with those studies. In future studies, epinephrine should not be used as a stressor for this cell line due to its inability to decrease cell viability

In future studies, oxidative stress could be measured with flow cytometry to understand if the cells were oxidatively stressed effectively. Viable aerobic cells produce reactive oxygen species such as O_2^-, OH, and peroxides but the cells also have an antioxidant defense system to reduce an excessive concentration of these reactive oxygen species (Eruslanov and Kusmartsev, 2010). In cells that are oxidatively stressed, the antioxidant defense mechanism is incapable of decreasing the ROS concentration because it is too high. Flow cytometry uses 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) as a intracellular probe to measure oxidative stress. DCFH-DA is non-fluorescent and is oxidatively converted to 2′-7′-dichlorofluorescein (DCF) which fluorescence at 530 nm (Eruslanov and Kusmartsev, 2010). The fluorescence of DCF measured at 530 nm with a flow cytometer is assumed to correlate with the concentration of hydrogen peroxide within the cell. High hydrogen peroxide concentration indicates high oxidative stress for the cell (Eruslanov and Kusmartsev, 2010). The oxidative stress for the epinephrine-

treated glial cells should be measured and if the cells are being oxidatively stressed, the cell viability increase would not be contributed to the lack of oxidative stress on the cells.

Trials performed had very different results, which can be seen from the large error bars in Figure 1. When performing the trials, it was noted that cells did not appear to be adhered to the bottom of the 96-well plate when treatment started. The cells formed disks and when adding solution or removing solution, the disks moved around in the media. Thus, when the media was removed viable cells and nonviable cells could have been removed. If the viable cells were removed, the results could be skewed. These observations could indicate the cells were not healthy prior to treatments or that they needed to be given more time to adhere in the 96-well plate. In future trials the cells should be left in the 96-well plates longer in order to become more established in the wells to avoid removing healthy cells.

From these results, ethynodiol diacetate, similarly to medroxyprogesterone acetate, does not appear to have protective capabilities previously seen in progesterone and segesterone acetate (Sitruk-Ware and El-Etr, 2013, Giatti et al., 2016). Ethynodiol diacetate is more structurally similar to testosterone, while segesterone acetate and medroxyprogesterone acetate are derivatives of progesterone (Giatti et al., 2016). These structural differences could perform different tasks within the body and may explain why segesterone acetate has protective capabilities, whilst ethynodiol diacetate does not. These differences, however, would not explain why medroxyprogesterone acetate does not have protective capabilities and segesterone acetate does, considering that both hormones are progesterone-derived. Further studies should explore trends in neuroprotective capabilities in testosterone related progestins and progesterone related progestogens and the mechanisms the hormones influence.

These treatments should be repeated to produce more consistent results to better assess to neuroprotective effects of ethynodiol diacetate. Further studies should be performed with a more

effective stressor and with cells more firmly adhered to the 96- well plate. Future work should also investigate the interactions between epinephrine and ethynodiol diacetate to better understand if they negatively regulate each other. Additionally, further studies could explore an increase in ethynodiol diacetate concentration to better understand if the hormone has neuroprotective effects at higher concentrations.

Ethynodiol diacetate is one of the main components of many oral birth control pills (Asi et al., 2016). The effects that ethynodiol diacetate have on the central nervous system are still unclear. The impact that ethynodiol acetate has on the central nervous system should be studied more extensively because many women use hormonal birth control over a period of decades. Negative effects that occur over many years could be a contributing factor to many neurodegenerative diseases, such as Alzheimer's, but positive effects over many years may protect against these diseases. For this reason, more studies need to be completed on the effects of progestogens on the central nervous system.

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Thesis Advisor_____ Date 5/8/19

Kathleen Hughes, Ph.D.

Committee Member_____ Date 05/08/19

Lauren King, Ph.D.

Honors College Dean_____ Date 5/9/19

Cindy Ticknor, Ph.D.